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Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*

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Abstract

Mobile genetic elements (MGEs) including plasmids and transposons are pivotal in the dissemination and persistence of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium*. Enterococcal MGEs have also been shown to be able to transfer resistance determinants to more pathogenic bacteria such as *Staphylococcus aureus*. Despite their importance, we have a limited knowledge about the prevalence, distribution and genetic content of specific MGEs in enterococcal populations. Molecular epidemiological studies of enterococcal MGEs have been hampered by the lack of standardized molecular typing methods and relevant genome information. This review focuses on recent developments in the detection of MGEs and their contribution to the spread of antimicrobial resistance in clinically relevant enterococci.

Keywords: antimicrobial resistance, enterococcus, insertion sequence, mobile genetic elements, plasmid, review, transposon

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Introduction

Enterococci are important nosocomial pathogens [1]. They are uniquely armed for the antibiotic era and express intrinsic reduced susceptibility to major classes of antimicrobial agents and biocides [2,3]. The high propensity of enterococci to acquire and express new resistance determinants further enhances their ability to sustain antibiotic selection, promoting gastrointestinal colonization and nosocomial infections by antibiotic-resistant enterococci [4].

Transferable antimicrobial resistance in enterococci was first described in the early 1970s [5–7]. The detection and molecular clarification of transferable high-level vancomycin resistance in *Enterococcus faecium* in the late 1980s have further fuelled our interest in the mechanisms and routes to antimicrobial resistance in enterococci [8–11]. Importantly, as residents of human and animal bowel flora, enterococci are in a position to acquire resistance genes from other commensals, which may subsequently proceed to other more pathogenic bacteria [12,13].

Molecular biological studies have elucidated complex functional properties of important mobile genetics elements (MGEs) involved in transferable resistance in enterococci [10,14,15]. However, there is a considerable gap in our knowledge on the molecular epidemiology of MGEs, their genetic content and composition, as well as their relative contributions to the spread of defined antimicrobial resistances. Recent progress in molecular typing methods and enterococcal genome information has provided new tools and necessary insights for filling this gap. This review focuses on MGEs involved in the spread and expression of clinically important antimicrobial resistance in enterococci and their potential contribution to the spread of hospital-adapted clonal lineages of *E. faecium* and *Enterococcus faecalis*.

Plasmids as Important Vehicles for Genetic Information in Enterococci

By definition, plasmids are semi-autonomously replicating extrachromosomal genetic elements. Differences in replica-

tion strategies and modular structures profoundly affect plasmid properties, such as size, copy number, host dependence and host range [16]. The essential backbones for successful plasmids include genetic modules encoding self-replication, stable inheritance and the ability to transfer between bacteria. Accessory plasmid content is integrated in between functional plasmid backbone modules and represents a huge reservoir of genetic variability, often with unknown functions, that is shared among different bacterial genera through horizontal gene transfer.

There are several criteria to classify plasmids in general and plasmids related to Gram-postive bacteria in particular. The mode of replication has been used to distinguish rolling circle replication plasmids and theta-replicating plasmids. In addition, plasmids that fail to co-reside in the same cell are grouped in incompatibility (Inc) groups [17,18]. Inc18 plasmids constitute a large group of enterococcal/streptococcal plasmids with a broad host range [19,20]. Pheromone-responsive plasmids represent a unique group of self-transferable (conjugative) narrow host range plasmids mostly described in *E. faecalis* [21]. pAD1, pAM373 and pCF10 are well-known examples of pheromone-responsive plasmids, where the conjugative process is initiated as a response to short peptide pheromones produced by pheromone-responsive plasmid-free recipient strains mediating intercellular aggregation and high-frequency DNA transfer. Recently, Weaver *et al.* [22] proposed a new family (RepA_N) of broadly distributed plasmids in Gram-positive bacteria encompassing pheromone-responsive plasmids of *E. faecalis*, as well as pRUM of *E. faecium*. Detailed sequence comparisons of the replication initiator protein suggest that the replicons have evolved along with their specific host, explaining their relatively narrow host range.

Plasmid replicon modules (replicons) have recently been used as targets of more simplistic methods for typing and epidemiological tracing of plasmids conferring antimicrobial resistance (R-plasmids). Other essential gene sets for plasmid survival, such as mobilization regions, have also been suggested as targets [23]. Given the modular evolution and genetic plasticity of plasmids, it is of note that schemes based on different core elements may not be congruent [16]. Carattoli *et al.* [18] developed a PCR-based plasmid typing method based on the replication regions from various plasmid incompatibility groups occurring in Enterobacteriaceae. A similar approach was recently described for the detection of plasmids from enterococci and other Gram-positive bacteria [24]. On the basis of 111 published sequences from Gram-positive bacteria, 19 replicon families (*rep*-family) and several unique replicons were identified. Using this PCR-based typing system, pCF10 (rep_9)-, Inc18 (rep_1 and rep_2)- and pUSA02 (rep_7)-related replicons were identified as being most prevalent in *E. faecalis* strains (n = 28), whereas Inc18 (rep_2)-, pRI1 (rep_{14})- and pRUM (rep_{17})-related replicons dominated in *E. faecium* strains (n = 51) of human and animal origin [24]. However, approximately 30% of the strains tested did not support any *rep*-detection, indicating the presence of unidentified *rep*-types.

The enterococcal-specific parts of the *rep*-detection system described by Jensen *et al.* [24] have been used by others. A recent study of an epidemiologically diverse collection of *E. faecium* strains (n = 93) revealed a high prevalence of Inc18 (rep_2)-, pRUM (rep_{17})- and pHTβ (rep_{unique})-related replicons [25]. The actual enterococcal typing scheme accounted for approximately 60% of the total number of plasmids visualized by S1-nuclease analyses. Interestingly, strains belonging to hospital-adapted clades (CC17-related) yielded a significant higher number of *rep* types and pRUM (rep_{17})-related replicons in particular, indicating a role in accessory plasmid DNA for promoting hospital adaptation. *Rep*-typing of extended *E. faecalis* strain collections has so far shown a dominance of pheromone-responsive plasmid ($rep_{8\ and\ 9}$)-, pS86 (rep_6)- and Inc18 ($rep_{1\ and\ 2}$)-related replicons (J. Sun, S. Xiaobo, T. Mikalsen, J. U. Ericson Sollid, A. Sundsfjord, unpublished observations). Other comprehensive studies include vancomycin-resistant *E. faecium* and *E. faecalis* strains causing hospital outbreaks in five continents, from 1986 to date (A. R. Freitas, M. V. Francia, L. Peixe, C. Novais, L. B. Jensen, R. J. Willems, F. Baquero, T. M. Coque, unpublished observations). Among *E. faecium*, mostly CC17-related, a high diversity of *rep* types could be identified; small [pB82 (rep_{11}), pRI1 (rep_{14}), pEF418 (rep_{18}), pCIZ2 (rep_{unique})] or medium to large plasmids [Inc18 ($rep_{1\ and\ 2}$), pRUM (rep_{17}), pHTβ (rep_{unique})], with *vanA* linked to Inc18- and pRUM-like plasmids in most cases. Vancomycin-resistant *E. faecalis* isolates belonging to major clonal complexes (CC2, CC9 and CC87) contained a lower diversity of plasmids, which were mostly associated with the narrow host pheromone-responsive pAD1 (rep_{9}) and Inc18-type ($rep_{1\ and\ 2}$) plasmids.

Linkage of clinically important resistance determinants to specific replicon types in enterococci is of interest for predicting potential transfer to other bacterial genera by conjugative broad host range plasmids. The application of pulsed-field gel electrophoresis of S1-nuclease-digested enterococcal DNA has proved very useful for the identification and sizing of enterococcal plasmids because they appear as linearized bands (5–400 kb) on a faint genomic background [25–27]. Physical linkage between defined plasmid *rep* types and resistance determinants can be visualized by co-hybridization analysis of linearized plasmid DNAs [25]. Co-hybridization

analysis and plasmid sequencing have shown linkage of the *vanA* operon to Inc18-, pHTbeta- and pRUM-related plasmids in *E. faecium* [25,28–31].

Plasmid genomic analysis in enterococci has been hindered by the comparably high number of extrachromosomal elements in many (especially clinical) strains; the tremendous size of several of these plasmids and the multicopy insertion sequence (IS) elements located on them complicating raw data and contig assembly. Sequencing of large multiresistance megaplasmids (>200 kb) are in progress, revealing the presence of multiple resistance determinants linked to known virulence-associated proteins (J. A. Laverde-Gómez, G. Werner, unpublished observations).

Transposable Elements Involved in Spread of Resistance in Enterococci

IS elements are the simplest transposable elements encoding only the enzyme(s) necessary for their own transposition. Transposons can be classified into three groups: (i) composite transposons; (ii) Tn*3* family transposons; and (iii) conjugative transposons [32]. A schematic presentation of enterococcal transposons from each of these groups is presented in Fig. 1. An extensive list of transposons containing specific resistance and virulence determinants described in enterococci is given in Table 1.

Composite transposons owe their intracellular mobility to the presence of flanking copies of IS of the same family that act together to move the DNA between them and have mostly been associated with high-level gentamicin resistance (HLGR) or *vanB1*-related glycopeptide resistance (Table 1). The Tn*3* family of transposons move (transpose) intracellularly within or between different replicons through a replicative mechanism promoted by a transposase (TnpA) and a resolvase (TnpR) [33]. Tn*3*-like transposons mediate high-level glycopeptide (VanA-type) and macrolide-lincosamide-streptogramin B (MLS_B) resistance in enterococci (Table 1). Conjugative transposons (CTns) have been classified as integrative conjugative elements (ICE) that encode all information necessary for their own excision, conjugation and integration into a new host [34]. CTns have been associated with resistance to tetracyclines and MLS-antibiotics as well as *vanB2*-related glycopetide resistance (Table 1). Members of the Tn*916*/Tn*1545*-family are considered to comprise classical conjugative transposons with a broad host range carrying clinically relevant resistance determinants between a larger diversity of bacterial genera (Table 1) [35,36]. Importantly, CTns are also able to co-transfer other plasmids and transposons, as well as facilitate transfer of large chromosomal fragments between strains [32].

Whole-genome sequencing and comparative genomic analysis have given insights into the mobile enterococcal DNA pool [38–40]. The IS appear to represent the major part of MGEs in clinical enterococcal isolates, and frequently found IS-families include IS*3*, IS*6*, IS*30*, IS*256* and IS*L3* [40–57]. Other IS element families found include IS*4*, IS*66*, IS*110*, IS*200*/IS*605*, IS*982*, IS*1182* and IS*1380* [40–42,49,51,58–60]. IS*16* is prevalent in hospital-adapted subpopulations of *E. faecium*, but has also been described in clinical *E. faecalis* strains and as part of pRUM-like plasmids [40–43].

We have limited knowledge concerning the prevalence and distribution of defined composite transposons. This is a result of their highly complex, dynamic and modular structure, which does not allow simplistic detection and typing methods. Recently, the ubiquitious presence of Tn*916* and Tn*1549*/*5382*-like elements has been demonstrated in oral and faecal human metagenomes from several countries using

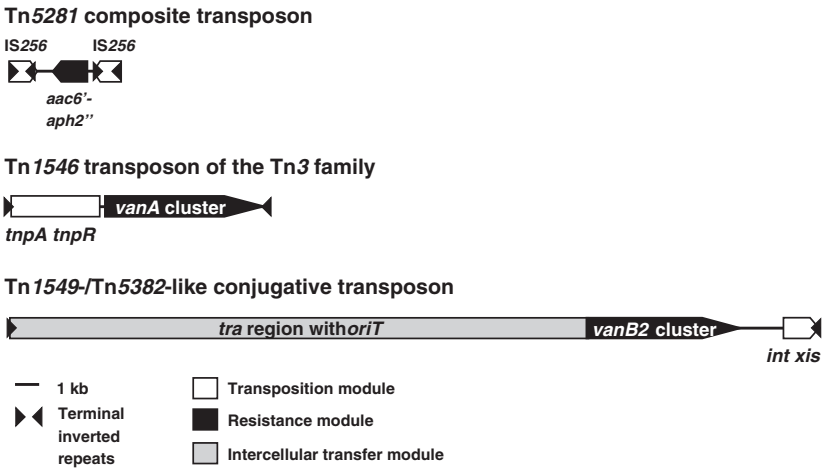

FIG. 1. Schematic presentation of transposons from each of the three transposon groups transferring resistance genes in enterococci. *aac6′-aph2″*, aminoglycoside-6′-*N*-acetyltransferase-2″-*O*-phosphoryltrasferase; *tnpA*, transposase; *tnpR*, resolvase; *vanA*/*vanB2* cluster, clusters conferring vancomycin resistance; *tra* region, region containing transfer genes; *oriT*, origin of transfer; *xis*, excisionase; *int*, integrase.

TABLE 1. Transposons containing antimicrobial resistance or virulence genes found in enterococci

Tn*3* transposon family

Tn designation(s)	Size (kb)	Replication and resolvation module	Resistance (gene)	Host range[a]	Reference
Tn*917* (Tn*551*) Tn*3871*	5,3 5,1	TnpA transposase (*tnpA*) and TnpR resolvase (*tnpR*)	Macrolides, lincosamides and streptogramin B (*ermB*)	*Bacillus*, *Enterococcus*, *Lactococcus*, *Listeria*, *Paenibacillus*, *Staphylococcus*, *Streptococcus*	[157–159]
Tn*1546* and variants	11	TnpA transposase (*tnpA*) and TnpR resolvase (*tnpR*)	Glycopeptides (*vanA*)	*Enterococcus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus circulans*, *Oerskovia turbata*, *Arcanobacterium haemolyticum*, *Paenibacillus*, *Rhodococcus*	[10,13]

Composite transposons

Tn designation(s)	Size (kb)	Flanking IS elements	Resistance (gene)	Host range[a]	Reference
Tn*5281* (Tn*4001*), Tn*3706* and variants of these	4,7	Inverted copies of IS*256* or IS*257*	Aminoglycosides (*aac(6′)-aph(2″)*)	*Enterococcus*, *Mycoplasma*, *Staphylococcus*, *Streptococcus*	[124,160–165]
Tn*924*	27	IS*257*-like	Aminoglycoside (*aac(6′)-aph(2″)*)	*Enterococcus faecalis*	[166]
Tn*1547*	64	IS*16* and IS*256*-like in a direct orientation	Vancomycin (*vanB1*)	*Enterococcus faecalis*	[43]
Tn*5384*	26	Directly repeated copies of IS*256*	Aminoglycosides (*aac(6′)-aph(2″)*), erythromycin (*ermB*), mercuric chloride (*merX*)	*Enterococcus faecalis*	[167,168]
Tn*5385*	Ca 65	Directly repeated copies of IS*1216*	Aminoglycosides (*aac(6′)-aph(2″)*), erythromycin (*ermB*), mercuric chloride (*mer*), streptomycin (*aadE*), tetracycline-minocycline (*tetM*), penicillin (*blaZ*)	*Enterococcus faecalis*	[69,169]
Tn*5405* and variants	12	Inverted copies of IS*1182*	Aminoglycosides (*aphA-3*, *aadE*), streptothricin (similar to *sat4*)	*Enterococcus faecium*, *Staphylococcus*	[60,170]
Tn*5482*	26–30	IS*1216V*	Glycopeptides (*vanA*)	*Enterococcus faecium*, *Enterococcus faecalis*	[44]
Tn*5506*	39	IS*1216V2* in inverted direction of IS*1216V1* with IS*1252* insertion	Glycopeptides (*vanA*)	*Enterococcus faecium*	[50]

Conjugative transposons (ICEs)

Tn designation(s)	Size (kb)	Recombination module	Resistance/virulence (gene)	Host range[a]	Reference
Tn*916*, Tn*918*, Tn*919*, Tn*920*, Tn*925*, Tn*3702*, Tn*5031*, Tn*5032*, Tn*5033*, Tn*5381*, Tn*5383*	15–23	Tn*916* tyrosine recombinase/integrase (*int*) and excisionase (*xis*)	Tetracycline-minocycline (*tetM*)	Species within the following genera: *Acholeplasma*, *Acinetobacter*, *Actinobacillus*, *Alcaligenes*, *Bacillus*, *Butyrivibrio*, *Citrobacter*, *Clostridium*, *Enterococcus*, *Erysipelothrix*, *Escherichia*, *Fusobacterium*, *Granulicatella*, *Haemophilus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Mycoplasma*, *Neisseria*, *Peptostreptococcus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Thermus*, *Ureaplasma*, *Veillonella*	[171–179]
Tn*1545* and variants	Ca 25	Tyrosine recombinase/ integrase (*int*) and excisionase (*xis*) related to those from Tn*916*	Tetracycline-minocycline (*tetM*), macrolides, lincosamides and streptogramin B (*ermB*), kanamycin (*aphA-3*)	Species within the following genera: *Bacillus*, *Bacteroides*, *Clostridium*, *Enterococcus*, *Escherichia*, *Eubacterium*, *Lactococcus*, *Listeria*, *Neisseria*, *Roseburia*, *Staphylococcus*, *Streptococcus*, *Ureaplasma*	[180,181]
Tn*6009*	18	Tyrosine recombinase/ integrase (*int*) and excisionase (*xis*) related to those from Tn*916*	Tetracycline-minocycline (*tetM*), mercury (*mer*)	*Enterococcus*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Streptococcus*	[182]

TABLE 1. (Continued)

Conjugative transposons

Tn designation(s)	Size (kb)	Recombination module	Resistance/virulence (gene)	Host range[a]	Reference
Tn*5382*,Tn*1549* and variants of these	27–34	Tyrosine recombinase/ integrase (*int*) and excisionase (*xis*) related to those from Tn*916*	Vancomycin (*vanB2*)	*Enterococcus faecium, Enterococcus faecalis, Enterococcus gallinarum, Enterococcus hirae, Clostridium, Streptococcus, Ruminococcus, Eggerthella lentha*	[42,63,64,104, 105,108,183]
Tn*5386*	29	Tyrosine recombinase/ integrase (*int*) and excisionase (*xis*) related to those from Tn*916*	Lantibiotics (*spa*), putative surface exposed collagen adhesion protein	*Enterococcus faecium*	[184]
OG1RF conjugative transposon homologue	Ca 49	Putative phage-related integrase, lacks excisionase homologue	Putative adhesin proteins	*Enterococcus faecalis*	[39]
Tn*6000*/EfcTn*1*	33	Excisionase and integrase related to those from *S. aureus* pathogenicity islands SaPIbov and SaPIbov2	Tetracycline (*tetS*)	*Enterococcus faecalis, Enterococcus faecium, Enterococcus casseliflavus, Enterococcus* spp.	[36,185; C. Novais, A. Freitas, F. Baquero, L. Peixe, M. Zervos, A. Roberts, T. M. Coque, unpublished observations
CW*459*TetM/Tn*5801* (Tn*6104*)	Ca 25	Integrase not related to Tn*916*	Tetracycline-minocycline (*tetM*)	*Enterococcus faecium, Enterococcus faecalis, Enterococcus* spp., *Staphylococcus aureus, Clostridium perfringens*	C. Novais, A. Freitas, F. Baquero, L. Peixe, T. M. Coque, unpublished observations
V583 *vanB1*-containing element	55	TndX-like large serine recombinase	Vancomycin (*vanB1*)	*Enterococcus faecalis*	[38]
Tn*5397* and variants	Ca 21	TndX-like large serine recombinase	Tetracycline-minocycline (*tetM*)	*Enterococcus faecium, Enterococcus hirae, Clostridium difficile, Bacillus subtilis, Streptococcus*	[186,187]
Tn*950*	47	Unknown, not homologous with Tn*916*	Erythromycin	*Enterococcus faecium*	[188]

[a]Host range includes both hosts into which the elements have been introduced and hosts in which they occur naturally.

integrase-specific probes for detection [61,62]. Detection of the most common CTns associated with tetracycline, macrolide or chloramphenicol resistance revealed the wide spread of sequences related to Tn*916*/Tn*1545,* Tn*5397*, and also CW*459*TetM/Tn*5801*and CTn*6000* among enterococci from different sources (C. Novais A. Freitas, F. Baquero, L. Peixe, M. Zervos, A. Roberts, T. M. Coque, unpublished observations), while Tn*1549*/*5382*-like elements are the most common vehicle for dissemination of VanB-type glycopeptide resistance in enterococci [42,63–66].

Transposable Elements: Contribution to Genome Plasticity and Phenotypic Variation

Genomic comparisons of host-adapted and less host-adapted species suggest a higher prevalence of mobile elements among the latter, specifying a comparably high genomic stability in very well host-adapted species and a comparably high genomic plasticity in variants with changing hosts or approaching new ecological niches [67]. The latter may be true for the recently emerging hospital-adapted clonal types of *E. faecium* and *E. faecalis* and may explain their large content of IS and other mobile elements among their genomes compared to other representatives, also within the same species (*E. faecalis* OG1RF/Symbioflor 1) [39,68]. Transposable elements may contribute to genome plasticity by several mechanisms. Multiple copies of related transposable elements are substrates for homologous recombination within and between different DNA elements mediating rearrangements in chromosomal and plasmid DNA [50,69,70]. Moreover, transposable elements may alter the expression of genes either through disruption of genes by insertion within the coding region or by integrations in the promoter region, either disrupting the existing promoter or providing a new and more efficient promoter. Enterococcal ISs found to contribute to the formation of functional promoters include IS*3*, IS*30*, IS*256* and IS*982* [71]. IS*1542* and IS*1191,* both belonging the IS*256* family, have been shown to contribute to constitutive expression of the *vanA* operon and to a higher level of biofilm formation in a virulent *E. faecalis*, respectively [45,47]. Moreover, the constitutive expression of vancomycin resistance in *vanD* isolates can be a result of IS*Efa4* (IS200/IS*605* family) insertion in the *vanD* operon regulator component determinant $vanS_D$ [59] and IS*19* (IS*982* family), disrupt-

ing the *ddl* gene encoding the housekeeping D-Alanine-D-Alanine ligase. Absence of D,D-carboxypeptidase activity in an *E. faecium* strain occurred as a result of IS*Efa9* (IS*3* family) insertion in $vanY_D$ which contributes to lowering the glycopeptide resistance level [49].

MGE and their Contribution to Specific Resistance Phenotypes

Transferable resistance to vancomycin

Subsequent to its first description [8,9], the vancomycin resistance operon (*van*) alphabet has expanded to eight in enterococci, namely the aquired *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL* [72] and *vanN* [73] in addition to the intrinsic *vanC* genotype in *E. gallinarum* and *E. casseliflavus*. Their characteristics and species distribution are summarized in Table 2. The *vanA* and *vanB* genotypes are the most commonly encountered forms of acquired glycopeptide resistance and have primarily been reported in *E. faecium* and *E. faecalis*. The mechanisms of resistance involve modification of the vancomycin-binding target by synthesis of peptidoglycan precursors with peptide sidechains that terminate in D-lactate (*vanA*, *vanB* and *vanD*) or D-serine (*vanC*, *vanE*, *vanG*, *vanL* and probably *vanN*) for which vancomycin has lower affinity than for the normal D-alanine sidechain terminus [74–77]. Detailed descriptions are provided in recent reviews [78,79].

The *vanA* gene cluster is carried by Tn*1546*, a Tn*3* derivative that is most often found as a part of nonconjugative or conjugative plasmids [10,44,50,80]. Broad host range IncI8 plasmids have been associated with transfer of Tn*1546* from enterococci to *Staphylococcus aureus* [81]. The *vanB* operon can be transferred between enterococci as part of large conjugative chromosomal elements or by conjugative plasmids [42,43,63,64,82–89]. The *vanB* ligase gene has been divided into three subtypes, *vanB1-3*, based on nucleotide sequence differences [90–92]. The *vanB2* subtype as an integral part of Tn*1549*/Tn*5382*-like conjugative transposons [42,63–66] is the most widespread *vanB* type in clinical enterococci [42,65,80,89,93–100]. Interestingly, the first description of a *vanB2*-Tn*1549*-like element in pheromone-responsive (pCF10-like) plasmids in *E. faecalis* strains was recently reported from Japan [89]. The occurrence of *vanB1* has only been described for single isolates as an integral part of putative ICE or composite transposons [38,43].

A high prevalence of the *vanB2* gene has been observed in community and hospital human faecal specimens in the

TABLE 2. Glycopeptide resistance due to *van*-type gene clusters

	Aquired							Intrinsic
Resistance level	**High**	**Variable**	**Moderate**	**Low**				**Low**
Type	**VanA**	**VanB**	**VanD**	**VanE**	**VanG**	**VanL**	**VanN**	**VanC**
MIC in mg/L:								
Vancomycin	≥16	≥4	≥64	6–32	12–16	8	8	2–32
Teicoplanin	>8	0,5-1	4–64	0,5	0,5			0,5-1
Expression	Inducible	Inducible	Constitutive/ Inducible (*vanD2*)	Inducible/ (Constitutive)	Inducible			Constitutive/ Inducible
van ligase gene	*vanA*	*vanB1-B3*	*vanD1-5*	*vanE*	*vanG1-2*	*vanL*	*vanN*	*vanC1-C3*
Modified target	D-alanine-D-lactate	D-alanine-D-lactate	D-alanine-D-lactate	D-alanine-D-serine	D-alanine-D-serine	D-alanine-D-serine	D-alanine-D-serine?	D-alanine-D-serine
Conjugative transfer	Yes	Yes	No	No	Yes	No	Yes	No
Location	Plasmid/chromosome on transposon(s)	Plasmid/chromosome ± transposon/ICE[a]	Chromosome	Chromosome	Chromosome on possible ICE	Chromosome?	Plasmid	Chromosome
Distribution	*Enterococcus faecium* *Enterococcus faecalis* *Enterococcus avium* *Enterococcus casseliflavus* *Enterococcus durans* *Enterococcus gallinarum* *Enterococcus hirae* *Enterococcus mundtii* *Enterococcus raffinosus* *Staphylococcus aureus* *Bacillus circulans* *Oerskovia turbata* *Arcanobacterium haemolyticum* *Paenibacillus* *Rhodococcus*	*Enterococcus faecium* *Enterococcus faecalis* *Enterococcus casseliflavus* *Enterococcus durans* *Enterococcus gallinarum* *Enterococcus hirae* *Staphylococcus epidermidis* *Streptococcus* *Clostridium* *Ruminococcus* *Eggerthella*	*Enterococcus faecium* *Enterococcus faecalis* *Enterococcus avium* *Enterococcus gallinarum* *Enterococcus raffinosus* Non-enterococcal faecal flora	*Enterococcus faecalis*	*Enterococcus faecalis* Non-enterococcal faecal flora	*Enterococcus faecalis*	*Enterococcus faecium*	*Enterococcus gallinarum – vanC1* *Enterococcus casseliflavus – vanC2/3*

[a]ICE, integrative conjugative element.

absence of cultivable vancomycin-resistant enterococci (VRE) [101–103]. This could be explained by the presence of *vanB2*-Tn*1549*/Tn*5382*-like elements identified in other bacterial genera belonging to the normal intestinal flora, such as *Clostridium, Ruminococcus, Eggerthella* and *Streptococcus* [104–106]. The high rates of non-enterococcal *vanB* in faecal samples result in a low predictive positive value for VRE using PCR-detection of *vanB* directly from faecal samples [103], whereas detection of *vanA* is considered highly specific [107]. These observations are also consistent with the recent description of Tn*1549* integrase in five of six faecal metagenomes from several European countries [62]. Furthermore, conjugative transfer of *vanB2*-Tn*1549*/Tn*5382*-like elements from *Clostridium symbiosum* to enterococci in the gut of gnotobiotic mice was recently described during vancomycin selection [108]. Thus, *vanB2* genes are already part of the normal faecal flora, and treatment with vancomycin might select for accidental transfer events to relevant human Gram-positive pathogens including enterococci. Indeed, rates of infections with *vanB*-type VRE (mainly *E. faecium*) are increasing in several European countries [109]. Most worrying is the finding of *vanA*-type vancomycin resistance in methicillin-resistant *S. aureus* (MRSA): VRSA. A detailed description of VRSA is provided elsewhere [81]. *S. aureus* may coexist with VRE in the gastrointestinal tract or in superficial wounds, providing a likely reservoir for development of VRSA [110]. Most recent outbreaks and clinical infections with VRE worldwide are a result of genetic lineages of hospital-adapted *E. faecium* [57,96,100,111–116] that have aquired several virulence and resistance determinants, increasing its ability to survive and cause disease in nosocomial environments [117–122].

High-level resistance to aminoglycosides

Acquisition of HLGR abolishes the synergistic bactericidal effect in combination with cell wall active agents in the treatment of severe enterococcal infections. The HLGR phenotype is most commonly associated with the bifunctional enzyme AAC(6′)-Ie-APH(2″)-Ia and the APH(2″)-I-family. The composite transposon Tn*5281* (IS*256*-related) or its variants have been shown to harbor the *aac(6′)-Ie-aph(2″)* determinant as part of conjugative enterococcal plasmids [123,124]. pMG1 is a fully sequenced conjugative pHTbeta-like 65-kb plasmid from *E. faecium* containing a Tn*5281*-like element [31]. pMG1-related replicons have been associated with transferable VanA-type vancomycin resistance and HLGR in *E. faecium* in the USA, Japan, the UK and the Netherlands [125,126]. HLGR in invasive *E. faecium* and *E. faecalis* strains in European countries is increasing [127,128; http://www.rivm.nl/earss/].

Linezolid resistance

Linezolid is an oxazolidinone antibiotic used in the treatment of infections caused by multidrug-resistant enterococci. Resistance, still uncommon in enterococci, is associated with mutations in central loop domain V of the 23S ribosomal RNA gene that affect the binding affinity between target and drug [129]. The first mutation in a single 23S allele is the key event in the development of linezolid resistance. Exchange of alleles via recombination occurs then readily, leading to a stepwise increasing resistance level [130]. If not all alleles have recombined, the resistance genotype is reversible. This type of acquired resistance is not transferable and spreads only clonally. Importantly, a new form of linezolid resistance has recently been described in staphylococci, Cfr, for chloramphenicol-florfenicol resistance [131]. The *cfr* determinant was originally described as plasmid-mediated and encodes a methylase that modifies the 23S rRNA ribosomal subunit, conferring cross-resistance to linezolid. The first *cfr* determinant was present in a genetic context including *ermB* and it was suggested to be of enterococcal origin. The *cfr* determinant has so far not been described in enterococci.

Resistance to quinopristin-dalfopristin (Q/D)

E. faecalis is intrinsically resistant to Q/D as a result of the presence of the *lsa* determinant, whose function remains to be established [132]. Several mechanisms of acquired Q/D resistance have been described in *E. faecium,* including 23S rRNA gene methylation, enzymatic inactivation of streptogramin A and efflux pumps [133,134]. Enterococcal plasmids harbouring *vat(D)* and *vat(E)* determinants encoding acetyltransferases and determinants for MLS_B resistance inactivating streptogramins A and B have been described [135–137].

Resistance to tigecycline

Only sporadic enterococcal isolates with phenotypic intermediate or borderline resistance (MIC = 0.5/≥1 mg/L) are reported in larger clinical trials. The molecular basis of tigecycline resistance in a single clinical *E. faecalis* isolate from an intensive care unit patient after prolonged tigecycline therapy has been investigated. Tigecycline resistance was stable in this epidemic, hospital-adapted strain type (ST6/clonal complex CC2). The mechanism of tigecycline resistance was not clarified [138].

Interactions between transferable antimicrobial resistance and pathogenicity

Several enterococcal plasmids have been shown to contain determinants associated with increased capacity for colonization and infection. In some instances, resistance genes and virulence determinants have been physically linked to the

same plasmid. The pheromone-responsive plasmid pAD1 encodes cytolysin [139] and aggregation substance (AS) [140]. pCF10 encodes AS [141], and the bacteriocin 21 gene (*bac21*) is found on pPD1 [142], *bac32* on pTI1 [143], *bac41* on pYI14 [144] and *bac43* on pDT1 [145]. The *bac41* gene was also recently described as genetically linked to *vanB2* on the pheromone-responsive and highly conjugative plasmid pMG2200 [89]. AS has been associated with *vanA*-containing pheromone-responsive plasmids in *E. faecalis* [146] and *E. faecium* [50,147]. Furthermore, large conjugative hyl_{Efm}-positive plasmids encoding VanA-type vancomycin resistance and/or resistance to erythromycin and high levels of gentamicin were recently found in *E. faecium* CC17-related strains [27,37]. These plasmids might also have a role in the success of the CC17 *E. faecium* genotype.

Persistence of R plasmids in the absence of antibiotic selection

Plasmids have systems that ensure stable inheritance. Interestingly, some R-plasmids contain plasmid addiction systems encoding labile anti-toxins and stabile and potent toxins that are able to kill daughter cells that do not inherit the plasmid during cell division (Fig. 2). Thus, once acquired, the R-plasmids ensure their own maintenance even in the abscence of antibiotic selection. Three functional plasmid addiction (TA; toxin–antitoxin) systems have been identified in enterococci; Axe-Txe in pRUM [41], epsilon-zeta in pVEF2 [28,148] and par/Fst system in pAD1 [149]. A prevalent genetic linkage between the pRUM-replicon type and *axe-txe* has recently been demonstrated in *E. faecium* strains, suggesting a successful plasmid backbone combination [25]. The stability of *vanA*-Inc18 plasmids in enterococci in the absence of glycopeptide selection has previously been linked to the epsilon-zeta TA-system [29,150]. The results of competitive fitness experiments of *vanA* plasmid-containing vs. recipient *E. faecium* cells revealed that environmental adaptation, *in vivo* gene transfer and plasmid maintenance system(s) favoured long-term VRE persistence without antibiotic selection and compensate for the biological costs of possessing the resistance genes [151]. These earlier *in vitro* experiments accompany recent prevalence studies of VRE among animal reservoirs in the absence of a direct antibiotic selective pressure [152,153].

New protection mechanisms against foreign DNA – means to limit the spread of antibiotic resistance?

Clustered, regularly interspaced, short palindromic repeats (CRISPR) have been shown to provide immunity to foreign DNA (phages and plasmids) by direct sequence match [154]. Many CRISPR elements contain phage sequences that provide the host with viral resistance [155,156]. In addition, sequences homologous to the nickase gene in conjugative plasmids of methicillin- and vancomycin-resistant *S. aureus* (MRSA and VRSA) are also found in functional CRISPR elements, interfering with plasmid conjugation and transformation in *Staphylococcus epidermidis* [156]. By contrast, a BLAST search of seven fully sequenced *E. faecium* strains found no functional CRISPR elements, whereas prophage sequences were common (Van Schaik, personal communication). Almost no mobile genetic elements were found in the fully sequenced *E. faecalis* OG1RF that contains two CRISPR elements [39]. An association was noted between the presence of CRISPR and the absence of prophages in this particular strain in contrast to V583. The role of CRISPR in the pro-

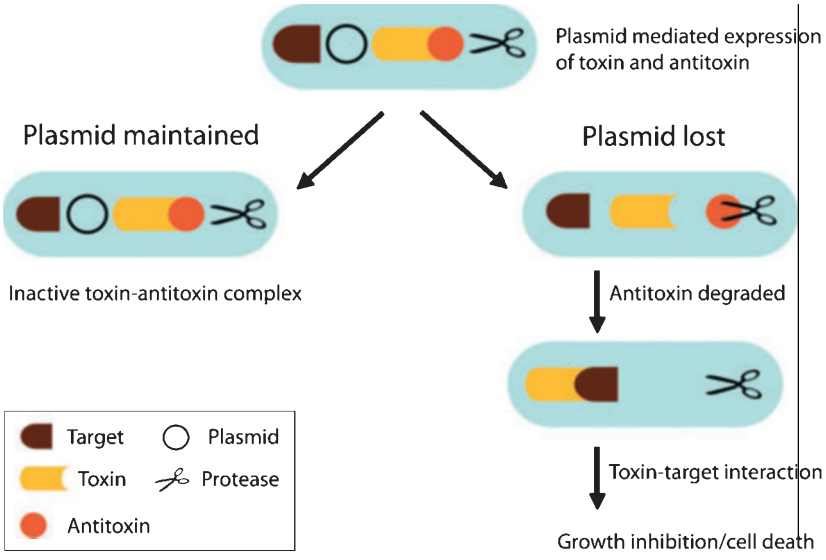

FIG. 2. Toxin–antitoxin (TA) pairs contribute efficiently to plasmid maintenance in a bacterial population. If TA-carrying plasmids are not inherited by the daughter cells after cell division, the less stable antitoxin is degraded by proteases and the toxin is left free to interact with its intracellular target, which leads to growth inhibition or cell death.

tection of foreign DNA in enterococci remains to be examined.

Concluding Remarks

Recent progress in enterococcal genome information and in the development of simplistic typing methods has allowed new insights into the prevalence, distribution and genetic content of MGEs in enterococci. This may allow an assessment of their relative contributions to the spread of defined resistance phenotypes and the potential risk for transfer to other bacterial genera by broad host range conjugative elements. The identification of mechanisms involved in maintenance of R-plasmids has provided information that, at least in part, can explain the persistence of defined transferable resistances in the absence of antibiotic selection. The potential use of genetically manipulated CRISPR interference mechanisms to limit the spread of antibiotic resistant enterococci needs to be examined.

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Transparency Declaration

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